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SOME ASPECTS OF NITROSAMINE FORMATION*

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In recent years, nitrosamines have caused considerable concern to the food industry, particularly to meat processors, because they are a potential health hazard. Why are nitrosamines a potential problem? Extensive research has shown that a wide range of nitrosamines have tumorigenic, mutagenic and carcinogenic properties. One of the most interesting features is that particular nitrosamines tend to induce tumors in different organs of test animals. Moreover, some of them can cause tumor formation after a single dose.

How can these nitrosamines form? One of the ways is by in vivo formation. There have been reports of the induction of malignant tumors in rats and mice by administration of sodium nitrite and secondary amines, such as: N, N'-dimethylurea (Sander; 1970), 2-imidazolidinone (Sander and Burkle, 1971), piperazine and N-methylaniline (Greenblatt, et al., 1971), morpholine (Greenblatt et al., 1971; Sander and Burkle, 1969) and N-methyl-benzylamine (Sander and Burkle, 1969). Treatment with sodium nitrite or secondary amine alone produced little or no effect, while the corresponding nitrosamine had a marked effect on the induction of tumors. Therefore, there is presumptive evidence that nitrosamines are formed in vivo. Alam, et al. (1971) confirmed the synthesis of N-nitrosopiperidine in rats from nitrite and piperidine, in vitro in gastric contents and in vivo in the stomach and small intestines, using TLC, GLC and mass spectrometric techniques. Sen et al. (1969) have demonstrated the in vitro formation of diethylnitrosamine, when diethylamine and sodium nitrite were incubated in the gastric juices of humans, dogs, cats, rabbits and rats. Human, cat and rabbit gastric juices (pH 1 to 3) were found to produce more nitrosamine than rat or dog gastric juices (pH 4 to 5.5). This suggests that cats and rabbits, whose gastric juices have a pH similar to that of man, would be more suitable test animals than the rat, provided their bacterial flora are similar. There has been only one report of nitrosamine formation in the human gastrointestinal tract. Sander and Sief (1969) fed diphenylamine and sodium nitrate to 31 fasting hospital patients. Examination of the gastric contents revealed that 11 of these patients showed great nitrite forming activity as well as a drop in pH to below 3, with subsequent diphenylnitrosamine formation. Diphenylamine was used in this study because its corresponding nitrosamine is non-carcinogenic. In a related experiment using rats, Alam et al. (1971) have claimed that nitrosopiperidine is synthesized from nitrate and piperidine in vitro in gastric juice and in vivo in the stomach and small intestine. In addition,

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Sander (1968) reported that nitrosamines can be formed from nitrate and secondary amines by nitrate-reducing bacteria. Under certain conditions aromatic substituted nitrosamines give yields greater than 50%. This has been confirmed by other investigators (Hawksworth and Hill, 1971; Klubes and Jondorf, 1971). Hawksworth and Hill (1971) claim that some bacteria having no nitrate-reducing activity catalyze formation of nitrosamines from secondary amines and nitrite at neutral pH; a pH at which one would not expect nitrosamine formation. However, glucose appears to be a necessary ingredient in the bacterial growth medium. This suggests that the bacteria may convert the glucose to acids which would lower the pH of the mixture. The authors should have determined how much nitrite was formed under the conditions used and run controls to see how much nitrosamine was formed with and without bacteria. The weakly basic aromatic secondary amines are known to yield significant quantities of nitrosamine at pH's below 7. Therefore, the question of how much nitrosamine formation is due to bacterial catalysis or chemical reaction alone is still unknown.

In general, the studies mentioned suggest that nitrosamines may form under physiological conditions in the human stomach with nitrate and/or nitrite, providing the pH, bacteria, and the appropriate amines or their precursors are present. In addition to this, there is a possibility that nitrosamines may be formed in food products prior to ingestion, particularly during processing or cooking. This is borne out by the occasional finding of a nitrosamine in cured meat products.

A survey of frankfurters produced by eight national manufacturers was carried out by our laboratory. In samples from six of the eight companies the amount of dimethylnitrosamine (DMNA) detected was insignificant (< 10 ppb) or could not be confirmed. In samples of the seventh company, only the first of 12 was found to contain DMNA at 48 ppb concentration. The first sample of the eighth company contained 84 ppb DMNA, the next 20 were found to be negative before another sample was confirmed at 11 ppb concentration.

In view of the lack of information available on the effect of cure ingredients, processing and storage conditions on nitrosamine formation, a study was undertaken to determine the effect of nitrite concentration on DMNA formation in frankfurters. The processing study was performed three times, covering a wide range of NaNO_2 concentrations. The results of the three studies were similar. Arbitrarily a level of 10 ppb DMNA was considered significant. Using a normal 2 hour cooking and smoking schedule, at levels of NaNO_2 up to 750 ppm or 5 times the permissible level that can be added to comminuted meat products, no significant DMNA was found in the frankfurters. Concentrations of DMNA of 10 ppb or greater were found in franks made with NaNO_2 levels of 1500 ppm or higher. For most of the levels of added nitrite at which apparent or confirmed DMNA could be demonstrated, there was some tendency for an increase in DMNA concentration when the frankfurters were cooked and smoked an additional two hours. From this study it appears that the legal limit of 156 ppm NaNO_2 added (or 1/4 oz per 100 lb chopped meat) is insufficient to produce significant amounts of DMNA in frankfurters, under our processing conditions. This study alone, however, does not help explain why DMNA is found in some commercial samples

and not in others. On the basis of this study, one of the many variables that can be suggested as contributing to DMNA formation in the products is localized high concentrations of nitrite in emulsions due to inadequate mixing during processing.

Further research is continuing on the effect of combinations of other cure components with NaNO_2 on nitrosamine formation. The determination of the factor or factors that are major contributors to, or inhibitors of, nitrosamine formation may enable meat processors to modify their curing procedure yielding nitrosamine free products.

The chemistry of nitrosamine formation is important so that one may be able to understand how nitrosamines form in vivo and in food products. This may result in procedures to prevent their formation. Since the presence of DMNA has been confirmed in several cured meat samples and more recently, nitrosopyrrolidine in fried bacon, it is of interest to know how they can be formed. The secondary amines, dimethylamine and pyrrolidine were reacted with a five fold molar excess of sodium nitrite over a pH range of 1 to 7. Maximum nitrosamine formation for both compounds occurred at approximately pH 3.35. At the average pH of fresh meats, 5.6, very little nitrosamine formed. However, there may be a greater tendency for the in vivo formation of nitrosamines if pH was the only factor involved. This is due to the variability of the pH of the stomach, from about 1.5 to 7 depending on which part of the stomach is involved and whether it is in a fasting or non-fasting condition. The rate of DMNA formation is greater than that of nitrosopyrrolidine over the entire pH range. This may be due to the fact that pyrrolidine is a stronger base than dimethylamine.

Some work has been reported in the literature on the synthesis of nitrosamines from amino acids, which are free secondary amines. Lijinsky et al. (1970) have formed nitrosamines from sarcosine, proline and hydroxyproline and the corresponding cyclic amino acids, azetidine-2-carboxylic acid and pipercolic acid which are closely related to proline. In addition to proline, Sander (1967) has made the nitrosamine of ephedrine. Archer, et al. (1971) have formed nitrososarcosine from creatine which is an amino acid, but does not contain a free secondary amine group. These precursor compounds are constituents of plant and animal tissues. It is fortunate that most of these nonvolatile nitrosamines are weak carcinogens, since the potential for the formation of these compounds is greater than for the volatile nitrosamines when one considers the type of precursors available in a biological system like meat.

There is also the potential for formation of volatile nitrosamines by either decomposition of nonvolatile nitrosamines or amino acids to give amines which in turn can be nitrosated. Ender and Ceh (1971) have claimed to form nitrosopyrrolidine from proline and other volatile aliphatic nitrosamines from the amino acids glycine, sarcosine, alanine and valine. These amino acids and nitrite were encapsulated in starch and heated. The nitrosamines were determined by thin-layer chromatography which does not confirm these products ambiguously. Under the experimental conditions used, one

would expect browning and other decomposition products to form that may give erroneous positive nitrosamine results. Therefore, there is some doubt as to whether some of these nitrosamines did form.

Nitrosamines may come from compounds other than secondary amines, which are not usually found free in biological systems. We undertook a study to determine the contribution to DMNA formation of naturally occurring quaternary ammonium compounds and some of their related tertiary amines which can be derived by either dealkylation or rearrangement of the parent compound. The conditions used were similar to those encountered in processing frankfurters and related meat products. Of the naturally occurring quaternary compounds, neurine produced the largest amount of DMNA with betaine, choline, acetylcholine and carnitine considerably lower. All of them produced lower yields of nitrosamine than tetramethylammonium chloride. All of these quaternary ammonium compounds can form tertiary amine derivatives. The tertiary amines used produced 4000 to 20,000 times the DMNA than was formed from the parent quaternary ammonium compound and 2 to 5 times that formed from trimethylamine. 2-Dimethylaminoethyl acetate yields almost one-half as much DMNA as dimethylamine. These results have appeared in one of our recent publications (Fiddler et al., 1972). This work is significant in showing DMNA can be formed from naturally occurring precursor quaternary ammonium compounds, even at low concentrations under our experimental conditions. In addition to the present concern about nitrosation of secondary amines, tertiary amines and to a lesser extent quaternary ammonium compounds may represent a source of amines for nitrosation.

What does all this data on the chemical formation of nitrosamines mean? It appears that there are components present in meat which can react with nitrite to form nitrosamines under certain conditions which, at the present time, are not clearly defined.

In conclusion, in spite of all the research that is currently taking place on nitrosamines, there are still some things we need to know. It is of prime importance that serious definitive research be performed to determine whether nitrosamines form in vivo under conditions normally encountered and to determine the extent of preformed nitrosamines, including non-volatile nitrosamines, in the food supply. The major question now will be whether the concentrations of nitrosamines found are of toxicological significance to man.

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ROBERT P. DUDLEY: Now from the FDA we come to the subject of methodology of analyses. We are generally not concerned with parts per billion in our everyday analyses, but we are now. The proof of the existence of nitrosamines in our foods requires analytical techniques for which only a few have the capability. The FDA developed this capability, and Mr. Thomas Fazio had a lot to do with it. Analyzing for nitrosamines has been a major obstacle during the past two years, and to tell you more about this we have one of the most knowledgeable people we could find, Tom Fazio of FDA. Tom is a veteran of the Korean War, has a B.S. in chemistry from The City College of New York, did graduate work at New York University and Polytechnic Institute of Brooklyn, majoring in organic and polymer chemistry.

At the present time, he is Section Chief, in charge of the Organic Chemistry Section, Bureau of Food, Food and Drug Administration. Prior to joining FDA in 1966, he spent twenty years working as a research chemist in R&D with Fleischmann Laboratories (Standard Brands, Inc.), Schwarz Laboratories (U.S. Brewing Academy), American Machine and Foundry Co. (Moorehead Patterson Research & Development Division), and the Rexall Chemical Co. His fields of research and analyses have been in foods, beverages, drugs, cosmetics, tobacco, plastics and coatings, and he has published 25 analytical papers to date in these areas.

Tom, how do we find nitrosamines in our foods if, in fact, they really exist?